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(57) Abstract

The inventors present a new strategy for *de novo* sequencing using oligonucleotide probe arrays which we term Inference Sequencing by Hybridization (ISBH). The ISBH method characterises a target DNA with several small degenerate probe arrays and reconstructs the base sequence of the target via computer algorithm at 100 % accuracy. ISBH returns a target sequence as multiple non-overlapping fragments several hundred to several thousand bases in length. Target DNAs up to 100 kilobases long can potentially be sequenced in a single experiment. A hypothetical ISBH probe array and sequence reconstruction algorithm were designed and tested by computer simulation on 76 DNA sequences obtained from GenBank comprising a total of 2.45 million base pairs. ISBH performs optimally on target DNAs in the range of 15–45 kilobases that have low levels of repeated sequence. We believe that the ISBH approach has great potential to increase the speed of DNA sequencing.

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INFERENCE SEQUENCING BY HYBRIDIZATION

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BACKGROUND OF THE INVENTION

Procedures involving use of Sequencing by hybridization (SBH) are known to those skilled in the art, and have recently been demonstrated to be useful as a powerful alternative to electrophoretic methods for diagnostic DNA analysis [M. Chee *et al.*, Accessing Genetic Information With High Density DNA Arrays, *Science* 274, 610 (1996); J. Hacia *et al.*, Detection Of Heterozygous Mutations In BRCA1 Using High-Density Oligonucleotide Arrays And Two-Color Fluorescence Analysis, *Nature Genetics* 14, 441 (1996)]. Diagnostic SBH employs hybridization of a target DNA sequence to a tiled array of several thousand short oligonucleotide probes of known sequence [W. Bains *et al.*, A Novel Method For Nucleic Acid Sequence Determination, *J Theor Biol*, 135, 303 (1988); E. Southern *et al.*, Hybridization With Oligonucleotide Arrays, *Genomics*, 13, 1008 (1992)]. The pattern of hybridization, detected by fluorescence microscopy, indicates which oligonucleotides in the probe array are present in the target DNA. When this information is compared against a reference target sequence, the entire sequence of the target DNA can be reconstructed at high accuracy.

SBH, while offering great advantages in terms of throughput for diagnostic sequence analysis, suffers from the drawback that a different probe array must be tailored for each target DNA analyzed [M. Chee *et al.*, Accessing Genetic Information With High Density DNA Arrays, *Science*, 274, 610 (1996)]. For *de novo* sequencing, current SBH methods are not competitive with electrophoretic sequencing techniques

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that yield 600-1000 base pair read lengths per experiment [P. Pevzner *et al.*, Improved Chips For Sequencing By Hybridization, *J Biomolecular Struct Dyn*, 9, 399 (1991)]. Even under perfect experimental conditions, existing SBH designs cannot reconstruct a unique target sequence from hybridization data alone [P. Pevzner *et al.*, Towards DNA Sequencing Chips, In *19th Int Conf Mathematical Foundations of Computer Science*, Lecture Notes Iin Computer Science, Springer-Verlag, Berlin, Vol 841, pp. 143-158 (1994); P. Pevzner, Rearrangements Of DNA Sequences And SBH, *Computers Chem*, 18, 221 (1994)]. Without a reference sequence for comparison, *de novo* SBH is fundamentally limited because it acquires base sequence information at the cost of positional information. One knows exactly *which* subsequences (probe sequences) are present in the target DNA but not *where* they are located.

Subsequences must be arranged by examining how they overlap with one another. For example, octa-nucleotides are assembled into longer sequences by finding corresponding seven-base overlaps. The accuracy of reassembly is limited because any particular subsequence can occur more than once in the target DNA, leading to an ambiguity in the final reconstructed sequence [P. Pevzner *et al.*, Towards DNA Sequencing Chips, In *19th Int Conf Mathematical Foundations of Computer Science*, Lecture Notes Iin Computer Science, Springer-Verlag, Berlin, Vol 841, pp. 143-158 (1994); P. Pevzner, Rearrangements Of DNA Sequences And SBH, *Computers Chem*, 18, 221 (1994)]. *De novo* SBH designs typically use the complete set of all possible oligonucleotide probes of a given length [W. Bains *et al.*, A Novel Method For Nucleic Acid Sequence Determination, *J Theor Biol*, 135, 303 (1988); N. Broude *et al.*, Enhanced DNA Sequencing By Hybridization, *Proc Natl Acad Sci USA*, 91, 3072 (1994); R. Drmanac *et al.*, DNA Sequence Determination by Hybridization: A Strategy For Efficient Large-Scale Sequencing, *Science*, 260, 1649 (1993); R. Drmanac *et al.*, Sequencing Of Megabase Plus DNA By Hybridization: Theory Of The Method, *Genomics*, 4, 114 (1989)]; the use of longer probes increases reconstruction accuracy but requires the use of very large arrays (> 10^9 probes), since the number of required probes increases exponentially with probe length. Even assuming perfect hybridization,

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an SBH array containing all ~10^6 possible 10-mers would reliably be able to sequence only about 600 bp of target DNA in a single experiment. As longer target DNA sequences are attempted, the reconstruction accuracy drops precipitously. For a detailed discussion of SBH reassembly algorithms and their limitations see references [P. Pevzner *et al.*, Towards DNA Sequencing Chips, In *19th Int Conf Mathematical Foundations of Computer Science*, Lecture Notes Iin Computer Science, Springer-Verlag, Berlin, Vol 841, pp. 143-158 (1994); P. Pevzner *et al.*, Improved Chips For Sequencing By Hybridization, *J Biomolecular Struct Dyn*, 9, 399 (1991); P. Pevzner, Rearrangements Of DNA Sequences And SBH, *Computers Chem*, 18, 221 (1994)].

All *de novo* SBH strategies proposed thus far are direct methods, that is, they directly probe the target DNA with oligonucleotides whose sequences are then assembled into longer fragments. Since it is currently not feasible to manufacture a probe array containing more than approximately 10^6 tiled oligonucleotide probes, a direct *de novo* SBH approach cannot outperform electrophoretic sequencing in terms of read length and reaaccuracy.

IN THE FIGURES

The present invention will become better understood with reference to the following description, appended claims, and accompanying figures where:

Figure 1. Basic concepts underlying the design of an Inference Sequencing by Hybridization (ISBH) probe array. A target, in this case a four-digit phone number, is characterized by a degenerate probe array with a four-fold redundancy using only 64 probes. The probe array does not detect any digit directly, but the information gathered is sufficient to unambiguously infer the identity of the number. A probe array based on conventional SBH designs capable of acquiring the same information would require 10,000 probes, one for each phone number.

Figure 2. General scheme for ISBH. A long single-stranded target DNA is sheared into short oligonucleotides and hybridized to an ISBH probe array. The pattern of hybridization is used to create a set of degenerate 16-mers that characterize

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the target DNA. Information from this degenerate set is used by an inference algorithm to produce a set of explicit 16-mers. The set of explicit 16-mers produced by the inference algorithm contains all 16-mers actually present in the target DNA sequence as well as "false positive" 16-mers that are not in the target. A data reduction algorithm is then used to eliminate the false positives from the set of explicit 16-mers. The explicit 16-mers that remain after data reduction are then reassembled at high accuracy into contiguous sequence.

Figure 3. Design of the ISBH probe array of degenerate 16-mers used in this study. The array consists of 25 different probe groups. Each group pattern represents $2^{16} = 65,536$ degenerate 16-mers, for a total of 1,638,400 probes. Each probe in the array represents 65,536 explicit 16-mers. Under ideal conditions, a single target 16-mer will hybridize to exactly one probe in each probe group.

Figure 4. Example of how false positives are generated by inference. Tetranucleotides from a target DNA are characterized by an ISBH probe array of degenerate 4-mers with two-fold redundancy (R = A or G; Y = C or T; W = A or T; S = C or G). The inference algorithm generates all valid combinations of data from the probe array to produce a set of nine inferred tetranucleotides, six of which are false positives. In general, the number of false positives generated by inference decreases with the number of probes used in the ISBH array. If the ISBH array used in this example had 16 additional probes, then no false positives would have been generated.

Figure 5. Data reduction after inference for the ISBH array of degenerate 16-mers used in this study. Seventy-six target DNA sequences downloaded from GenBank comprising a total of 2.45 million bases were tested by computer simulation. The number of 16-mers generated by inference increases as a power law function of the number of different 16-mers in the target DNA (filled circles). Data reduction reliably eliminated all but a handful of the false positives for all target lengths investigated (open triangles), even when false positives comprised more than 99% of the inferred 16-mer set.

Figure 6. Absolute performance of the ISBH sequence reassembly algorithm. The reassembly algorithm returns a target DNA as several non-overlapping fragments

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in the range of several hundred to several thousand bases in length. The largest fragment reconstructed in this study was 28 kilobases, and fragments longer than 10kb were commonly observed. Reconstructed fragments always show 100% identity to some region of the target DNA.

Figure 7. Total target coverage in a simulated ISBH experiment. ISBH typically covers more than 95% of a target DNA in a single hybridization experiment in fragments that are longer than 500 bases.

Figure 8. Summary of ISBH simulation results. All lengths are in bases. Locus and definition for each sequence are shown exactly as they appear in GenBank. The number different 16-mers in a target is defined as the number of 16-mers which have different base sequences. The fraction of target 16-mers that are repeated is given by: 1 - [(number of different 16-mers in target)/(target length - 15)], which is a quantitative measure of repetitiveness of the sequence. The fraction of the inferred set that are false positive is given by: 1 - [(number of different 16-mers in target)/(number of 16-mers in inferred set)].

SUMMARY OF THE INVENTION

The present invention is directed to a method that satisfies the above mentioned problems by introducing a new SBH implementation for *de novo* sequencing, which we term Inference Sequencing by Hybridization (ISBH). The basic concepts underlying ISBH are illustrated in Figure 1 with an example of how to determine the last four digits of a phone number without detecting any digit directly. A conventional SBH strategy would require $10^4 = 10{,}000$ probes (one for each phone number), whereas the ISBH approach gathers the same information using only $4 \times 2^4 = 64$ degenerate probes. The digit groupings in Figure 2 are analogous to familiar base groupings such as purine/pyrimidine (R/Y), amino/keto (M/K), and weak/strong (W/S).

ISBH is an indirect strategy that uses several small arrays (65,536 probes each) to closely approximate the information that would be gathered from a single SBH array containing ~4.3 billion probes (Figure 2). Our strategy relies on degenerate probe arrays that are similar to binary SBH arrays proposed by Pevzner *et. al.* [P. Pevzner *et*

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al., Towards DNA Sequencing Chips, In *19th Int Conf Mathematical Foundations of Computer Science*, Lecture Notes Iin Computer Science, Springer-Verlag, Berlin, Vol 841, pp. 143-158 (1994)]. Unlike conventional SBH, whose accuracy drops with increasing target length, we find that ISBH always reconstructs targets at 100% accuracy. ISBH returns a target sequence as non-overlapping fragments that range from several hundred to several thousand bases in length. The number of fragments increases with the length and repetitiveness of the target DNA, but in principle, any length of target can be sequenced in a single experiment. We have designed and empirically tested by computer simulation an ISBH array and reconstruction algorithm for use in *de novo* sequencing of targets up to 100 kilobases in length.

METHODS

The inventors have simulated the following laboratory experiment: 1) a single stranded target DNA of unknown sequence is sheared into overlapping oligomers 16 bases long. 2) These oligomers are hybridized to an ISBH degenerate probe array and this pattern of hybridization is detected. 3) the hybridization data is reconstructed by computer algorithm into contiguous sequence.

An ISBH probe array containing 1.64 million different degenerate 16-mers was designed. This array is 25-fold redundant - it consists of 25 groups of 2^{16}=65,536 (64K) different probe oligomers. Figure 3 shows the identity of each degenerate probe group in the array. Each group of 64K degenerate probes is capable of hybridizing to all possible explicit 16-mers. Thus, a single explicit 16-mer will, under ideal conditions, hybridize to exactly 25 different degenerate probes in the ISBH array.

Simulations of ISBH experiments and subsequent data analysis were performed on a Silicon Graphics Origin2000 supercomputer (Boston University Center for Computational Science) with code written in C as follows: 1) Each target DNA sequence was retrieved from GenBank (http:// *www2.ncbi.nlm.nih.gov/genbank*) and broken up into all possible component 16-mers. This set of 16-mers was then shuffled to destroy any positional information. 2) Each 16-mer from the target DNA was compared against all 1.64 million degenerate probes in the ISBH array, simulating

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ideal hybridization. If a 16-mer from the target was found to hybridize with one of the degenerate probes, then the hybridization was considered to be a signal arising from that particular probe in the ISBH array. 3) The 16-mers from the target DNA were then discarded, to account for the fact that in a real experiment, the target DNA is of unknown sequence. 4) The signals from the ISBH array were collected to produce a set of degenerate 16-mers, which is the non-repeated set of degenerate 16-mers present in the target DNA. We emphasize that the only data retained is the signal pattern from the ISBH array, as would be detected in an actual ISBH experiment. The degenerate set contains no information about the explicit base sequence of any 16-mer present or its position in the target DNA sequence.

Sequence Reconstruction

1) *Inference.* A set of explicit 16-mers is inferred from the set of degenerate 16-mers detected by the ISBH array. The inference is accomplished by testing every possible explicit 16-mer against the degenerate set. Any particular explicit 16-mer is included in the inferred set only if exactly 25 corresponding degenerate 16-mers are present in the data from the ISBH array. Under conditions of ideal hybridization, the inferred 16-mer set is always a superset of the set of 16-mers present in the target DNA sequence. The inferred set usually contains false positive 16-mers, ones which are not actually present in the original target DNA sequence. The number of these false positives increases with the length and repetitiveness of the target DNA (Figure 4).

2) *Primary Data Reduction.* Since the inferred set of explicit 16-mers contains an unknown number of false positives, a data reduction step is required to eliminate them. Every 16-mer in the inferred set is examined to determine if it overlaps by six bases at least one other 16-mer in the inferred set on both its 3' and 5' ends. If both overlaps are not found the 16-mer is discarded. This procedure is repeated iteratively on the resultant set of 16-mers, each time examining an overlap one base longer than was used for the previous iteration, until an overlap of fifteen bases is reached. The

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set that remains is then iterated using fifteen-base overlaps until four or fewer 16-mers are discarded at each iteration.

3) *Secondary Data Reduction.* All possible reconstruction ambiguities are eliminated by comparing the 3'-fifteen bases of each 16-mer with the 3'-fifteen bases of every other 16-mer in the set from step 2 above. If two or more 16-mers are found to have identical fifteen base 3'-ends, then they are all discarded from the data set. A similar procedure is used to compare the 5'-fifteen bases of the 16-mers and eliminate any duplication.

4) *Sequence Reassembly.* The 16-mers remaining in the inferred set are then assembled into longer sequences. This is done by comparing the 3'-fifteen bases of each 16-mer to the 5'-fifteen bases of every other 16-mer in the data set. If a match is found, then the two 16-mers are combined into a single 17-mer. The two terminal 15-mers on either side of this newly formed 17-mer are compared for overlap with the remaining 16-mers in the data set and the process is repeated until no more overlaps are found. To insure reconstruction accuracy, only fragments at least 100 bases in length were considered to be part of the target sequence.

RESULTS

Inference Algorithm

ISBH simulations were performed on 76 target sequences obtained from GenBank comprising a total of 2.45 million bases, ranging from 5 to 100 kilobases in length. The size of the inferred 16-mer pool increases as a power law function of the number of different 16-mers in the target DNA. Data reduction reliably eliminates all but a handful of the false positives for all target lengths investigated, even when false positives accounted for more than 99% of the inferred 16-mer pool (Figure 5) . The set of inferred 16-mers remaining after data reduction closely approximates the information that would be gathered from an SBH array containing all explicit 16-mers (~4.3 billion probes).

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Sequence Reconstruction

The ISBH reconstruction algorithm typically returns a target DNA as several non-overlapping fragments that are in the range of several hundred to several thousand bases in length. Reconstructed fragments always show 100% identity (by BLASTN) to some region of unknown position in the target DNA sequence. The largest single fragment reconstructed was 28 kb, and fragments longer than 10kb were commonly observed (Figure 6). In most cases, more than 95% of the target DNA was recovered in a simulated ISBH experiment (Figure 7). ISBH performs optimally on target DNAs having no repeated 16-mers, generally returning a handful of long (3-15 kb) fragments. Even on sequences with many repeated 16-mers, ISBH returns dozens of fragments shorter than 5 kb, which is five to ten times the performance of electrophoretic sequencing methods. Target DNAs longer than 50 kb tend to produce large numbers of false positives in the inference step, a few of which remain after data reduction. False positives introduce ambiguities during reassembly, leading to lower average lengths for reconstructed fragments. A comprehensive list of each sequence analyzed as well as a summary of the ISBH simulation results are shown in Figure 8.

DETAILED DESCRIPTION OF THE INVENTION

While this invention is satisfied by embodiments in many different forms, there will herein be described preferred embodiments of the invention, with the understanding that the present disclosure is to be considered exemplary of the principles of the invention and is not intended to limit the invention to the embodiments illustrated and described. The scope of the invention will be measured by the appended claims and their equivalents.

A benefit of the present invention, in contrast to conventional SBH, is that the inventor's ISBH method has the potential to sequence very long targets at high accuracy, using an oligonucleotide array of moderate size. The hypothetical ISBH array studied here could easily sequence 15-45kb of DNA in a single experiment. The ISBH method requires no electrophoresis, no information about the target DNA, and could be used for diagnostic as well as *de novo* applications. In a single experiment,

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ISBH could generate more sequence data than two dozen Sanger sequencing reactions after shotgun subcloning of a target DNA. In the best cases, each fragment reconstructed by the ISBH method can outperform electrophoretic methods by 28-fold. In the worst cases, ISBH performance is equivalent to electrophoretic methods. ISBH reconstruction of a single target DNA generally required less than 10 minutes of supercomputer time to complete. The computational complexity of the inference step is of order N^2, while the data reduction and reassembly steps are of order N log(N). Sequence reconstruction using a highly streamlined ISBH algorithm running on a typical desktop computer could be completed in a few hours.

For DNA of random sequence, a given 16-mer should appear once every $4^{16} \approx 4.3 \times 10^9$ bases, a 15-mer once in $4^{15} \approx 10^9$ bases, and a 14-mer once in $4^{14} \approx 2.7 \times 10^8$ bases. We note however, that DNAs from a wide variety of organisms in the range of 10-100 kb typically have hundreds or thousands of repeated 16-mers. As shorter subsequences are examined, the number of repeated subsequences increases dramatically. For example, the 48.5 kb genome of bacteriophage lambda, which has no repeated 16-mers, has a single repeated 15-mer, and ten distinct 14-mers appearing more than once. This would suggest that any form of *de novo* SBH using oligonucleotide probes shorter than 16 bases will perform poorly on target DNAs longer than a few kilobases. ISBH appears to perform optimally both in terms of absolute read lengths and relative target coverage on DNAs in the range of 25kb that have small numbers of repeated 16-mers. ISBH is a scalable technique - the number of false positives generated by inference increases as the number of probes used in the ISBH array decreases. An ISBH array smaller than the one examined here (*e.g.*, 12 probe groups using $12 \times 2^{16} = 7.86 \times 10^5$ probes) would still sequence with 100% accuracy, but would return a target as shorter fragments.

While ISBH under ideal conditions would appear to provide an enormous gain for *de novo* sequencing over conventional SBH and electrophoretic methods, several daunting technical obstacles remain. Each degenerate probe is actually a mixture of many individual probes that are bound to the same area in an SBH array. The binding capacity of such a degenerate probe is greatly reduced in comparison to a pure

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individual probe - for the hypothetical ISBH array in this study, the complexity of each degenerate probe is 65,536. Such a high probe complexity may mean that accurate physical hybridization cannot be achieved with a high signal to noise ratio. Possible solutions to this problem include the use of base analogs to decrease probe complexity or the addition of an enzymatic step (*e.g.*, ligation) to augment the accuracy of simple physical hybridization.

Noise contamination of the data set, particularly in terms of false negatives, must be studied in greater detail. False positives are easily dealt with in the data reduction step, but false negatives (target 16-mers that never appear at all in the inferred data set) will have the effect of lowering the mean fragment length during reconstruction. Aberrant hybridization also increases the complexity of data processing needed for reliable sequence reconstruction; the upper bound for robust sequence reconstruction from an actual ISBH implementation is likely to be somewhat lower than the ideal situation presented here.

An ISBH sequencing approach would be very effective for rapid analysis of viral and bacterial genomes which are essentially non-repeating. Sequencing of double-stranded target DNAs by ISBH is also possible, as is sequencing of a mixture of targets. For double-stranded DNA, ISBH performance is equivalent to the case of a single-stranded DNA twice as long. If a double stranded target is cleaved by a restriction endonuclease before hybridization, ISBH will return the sequence of each restriction fragment. If this experiment is repeated using a restriction endonuclease with a different recognition site, then the fragments can be aligned relative to one another using standard contig reassembly algorithms. The potential of the ISBH strategy is so strong that we are now investigating strategies to implement it in practice.

Another embodiment of the ISBH Probe Array, Experimental, and Data Analysis Algorithm Design consists of the following:

Probe Array Design. The proposed array consists of 768K oligonucleotide probes divided into three groups: 1) all 256K possible 9-base single-stranded

sequences, 2) all 256K possible 9-base 5'-overhanging partial duplexes, and 3) all 256K possible 9-base 3'-overhanging partial duplexes.

Target Preparation. The target DNA must be single stranded - it may be prepared (for example) by long PCR of a double stranded target using one primer that is biotinylated at its 5' end to facilitate purification from the other strand. The biotinylated strand may be captured on streptavidin-coated beads or column.

Experimental Design. Eight distinct oligonucleotides in two groups are required as follows. Group I: 5'-ANNNNNNN-3', 5'-CNNNNNNN-3', 5'-GNNNNNNN-3', 5'-TNNNNNNN-3'. Group II: 5'-P-NNNNNNNA-Sdd-3', 5'-P-NNNNNNNC-Sdd-3', 5'-P-NNNNNNNG-Sdd-3', and 5'-P-NNNNNNNT-Sdd-3'. P denotes a phosphate group, Sdd denotes a 3'-dideoxy base connected by a phosphorothioate linkage. Sixteen separate reactions are then performed: each oligonucleotide from group I is combined with an oligonucleotide from group II and then hybridized to the single-stranded target under conditions favoring accurate base-pairing. After hybridization has occurred, the oligonucleotides that are still remaining in solution (for example) must be removed by size-exclusion column chromatography. DNA ligase (and all necessary cofactors) are added to the reaction mixture. After the ligation reaction, exonuclease III is added to the reaction mixture to destroy any unligated oligonucleotides that are still hybridized to the target.

Under ideal conditions, the ligation products will be 16-mers of the form: 5'-ANNNNNNNNNNNNNNA-Sdd-3' (and all 15 other permutations of the end bases). The ligation products from each of the sixteen reactions are then hybridized separately to a probe array as described above.

Data Analysis. Each of the sixteen probe array hybridization experiments described above generates the following data: a set of 9-mers from probe group 1, a set of 9-mers from probe group 2, and a set of 9-mers from group 3. The following algorithm is used to expand the data: compare each 9-mer from group 2 to each 9-mers from group 3. If the 9-mer from group 2 has the same 3' two bases as the 5' two bases of the 9-mer in group 3 (*i.e.*, they have a two-base overlap), then combine the two 9-mers to form a single 16-mer (concatenate the 3' seven bases of the group 3

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oligo to the 3' end of the group 2 oligo). This newly formed 16-mer is retained only if all eight of its 9-base subsequences are present in group 1. This analysis is performed for all probe array experiments and all retained 16-mers are collected into a single set. This set of 16-mers (which is the inferred set of explicit 16-mers from the target) is then subjected to the same data reduction and sequence reconstruction algorithms that we have previously described for ISBH.

Accordingly, this invention is not limited to the particular embodiments disclosed, but is intended to cover all modifications that are within the spirit and scope of the invention as defined by the appended claims.

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CLAIMS

What is claimed is:

1. A method of testing a nucleic acid target, comprising:

a) fragmenting a single-stranded target DNA into single-stranded target DNA fragments;

b) testing said fragments so as to generate a signal for each fragment;

c) calculating a first set of N-mers from said signals, each of said N-mers having a sequence with 3' and 5' ends;

d) comparing a portion of the nucleic acid sequence of each of said N-mers of said first set with a portion of the nucleic acid sequence of every other N-mer in said first set for sequence overlap; and

e) eliminating each N-mer that is found not to display said overlap, so as to create a second set of N-mers.

2. The method of Claim 1, further comprising the steps:

f) comparing a portion of the nucleic acid sequence of each of said N-mers in said second set with a portion of the nucleic acid sequence of every other N-mer in said second set for sequence overlap, wherein the portion compared has a length in bases defined by N-1; and

g) identifying an instance where said portion of one N-mer from said second set is found to overlap with said portion of another N-mer from said second set, thereby identifying first and second overlapping N-mers.

3. The method of Claim 1, wherein non-target DNA fragments are added to said single-stranded target DNA fragments prior to step (b).

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4. The method of Claim 2, wherein N is 16 and said portion compared in step (f) is fifteen bases in length.

5. The method of Claim 4, wherein said fifteen bases compared in step (f) are the 3'-fifteen bases of each 16-mer with the 5'-fifteen bases of every other 16-mer in said second set.

6. The method of Claim 5, further comprising the step (h) constructing a 17-mer from the combination of the sequences of said first and second overlapping N-mers of step (g).

7. A method of testing a nucleic acid target, comprising:

a) fragmenting a single-stranded target DNA into single-stranded fragments;

b) testing each of said fragments so as to generate a signal for each fragment;

c) calculating a first set of N-mers from said signals, each of said N-mers having a sequence with 3' and 5' ends;

d) comparing a portion of the nucleic acid sequence of each of said N-mers of said first set with a portion of the nucleic acid sequence of every other N-mer in said first set for sequence overlap;

e) eliminating each N-mer that is found not to display said overlap, so as to create a second set of N-mers;

f) comparing a portion of the nucleic acid sequence of each of said N-mers in said second set with a portion of the nucleic acid sequence of every other N-mer in said second set for sequence overlap, wherein the portion compared has a length in bases defined by N-1; and

g) identifying an instance where said portion of one N-mer from said second set is found to overlap with said portion of another N-mer from said second set, thereby identifying first and second overlapping N-mers.

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8. The method of Claim 7, wherein non-target DNA fragments are added to said single-stranded target DNA fragments prior to step (b).

9. The method of Claim 8, wherein N is 16 and said portion compared in step (f) is fifteen bases in length.

10. The method of Claim 9, wherein said fifteen bases compared in step (f) are the 3'-fifteen bases of each 16-mer with the 5'-fifteen bases of every other 16-mer in said second set.

11. The method of Claim 10, further comprising the step (h) constructing a 17-mer from the combination of the sequences of said first and second overlapping N-mers of step (g).

12. A method of testing a nucleic acid target, comprising:

a) fragmenting a single-stranded target DNA into single-stranded fragments;

b) adding non-target DNA fragments to said single-stranded target DNA fragments to create a mixture;

c) testing each of said fragments so as to generate a signal for each fragment;

d) calculating a first set of N-mers from said signals, each of said N-mers having a sequence with 3' and 5' ends;

e) comparing a portion of the nucleic acid sequence of each of said N-mers of said first set with a portion of the nucleic acid sequence of every other N-mer in said first set for sequence overlap;

f) eliminating each N-mer that is found not to display said overlap, so as to create a second set of N-mers;

9/28/2007, EAST Version: 2.1.0.14

g) comparing a portion of the nucleic acid sequence of each of said N-mers in said second set with a portion of the nucleic acid sequence of every other N-mer in said second set for sequence overlap, wherein the portion compared has a length in bases defined by N-1; and

h) identifying an instance where said portion of one N-mer from said second set is found to overlap with said portion of another N-mer from said second set, thereby identifying first and second overlapping N-mers.

13. The method of Claim 12, wherein N is 16 and said portion compared in step (g) is fifteen bases in length.

14. The method of Claim 13, wherein said fifteen bases compared in step (g) are the 3'-fifteen bases of each 16-mer with the 5'-fifteen bases of every other 16-mer in said second set.

15. The method of Claim 14, further comprising the step (i) constructing a 17-mer from the combination of the sequences of said first and second overlapping N-mers of step (h).

9/28/2007, EAST Version: 2.1.0.14

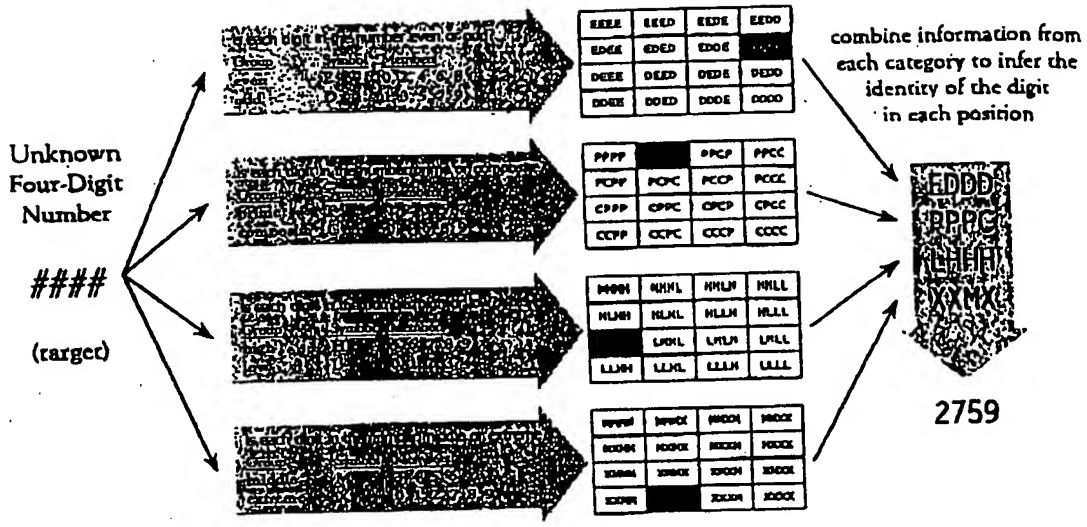

Unknown
Four-Digit
Number
####
(target)
combine information from
each category to infer the
identity of the digit
in each position
2759

Figure 1

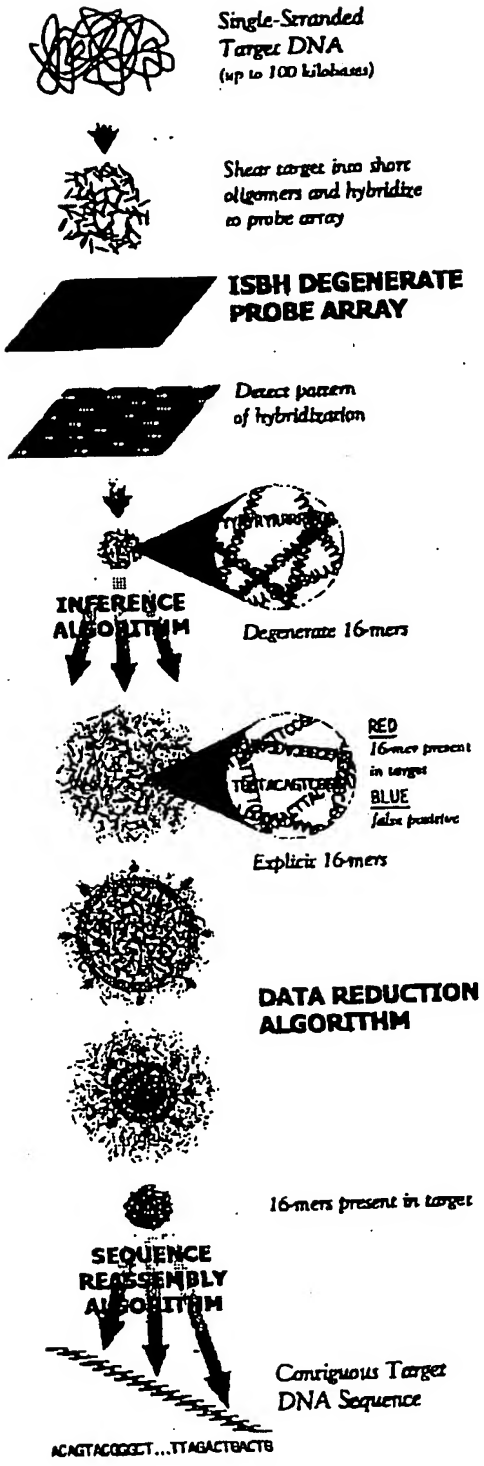

Single-Stranded
Target DNA
(up to 100 kilobases)
Shear target into short
oligomers and hybridize
to probe array
ISBH DEGENERATE
PROBE ARRAY
Detect pattern
of hybridization
INFERENCE
ALGORITHM
Degenerate 16-mers
RED
16-mer present
in target
BLUE
false positive
Explicit 16-mers
DATA REDUCTION
ALGORITHM
16-mers present in target
SEQUENCE
REASSEMBLY
ALGORITHM
Contiguous Target
DNA Sequence
ACAGTACGGGCT...TTAGACTGACTG

Figure 2

Degenerate Probe Group Pattern	*Example Probe*
$[A/B]_{16}$	ABAABBABABABABBA
$[C/D]_{16}$	DCDCDDCCCDCDCCDC
$[G/H]_{16}$	GHHHGGGHGHHGHHHG
$[T/V]_{16}$	TVTTTTVTVVVTTVTT
$[R/Y]_{16}$	RYRRYYRRYYRRYYRR
$[M/K]_{16}$	MKKKMMKMMMMKKMKM
$[W/S]_{16}$	SSWSWSSWWSWWSWSW
$[R/Y]_8[M/K]_8$	RRRRRRRRMMMMMMMM
$[M/K]_8[R/Y]_8$	MMMMMMMMRRRRRRRR
$[M/K]_8[W/S]_8$	MMMMMMMMWWWWWWWW
$[W/S]_8[M/K]_8$	WWWWWWWWMMMMMMMM
$[R/Y]_8[W/S]_8$	RRRRRRRRWWWWWWWW
$[W/S]_8[R/Y]_8$	WWWWWWWWRRRRRRRR
$[M/K]_4[R/Y]_8[M/K]_4$	MMMMRRRRRRRRMMMM
$[W/S]_4[R/Y]_8[W/S]_4$	WWWWRRRRRRRRWWWW
$[W/S]_4[M/K]_8[W/S]_4$	WWWWMMMMMMMMWWWW
$[R/Y]_4[M/K]_8[R/Y]_4$	RRRRMMMMMMMMRRRR
$[R/Y]_4[W/S]_4[R/Y]_4$	RRRRWWWWWWWWRRRR
$[M/K]_4[W/S]_8[M/K]_4$	MMMMWWWWWWWWMMMM
$([R/Y][M/K])_8$	RMRMRMRMRMRMRMRM
$([W/S][M/K])_8$	WMWMWMWMWMWMWMWM
$([R/Y][W/S])_8$	RWRWRWRWRWRWRWRW
$([R/Y]_4[M/K]_4)_2$	RRRRMMMMRRRRMMMM
$([R/Y]_4[W/S]_4)_2$	RRRRWWWWRRRRWWWW
$([W/S]_4[M/K]_4)_2$	WWWWMMMMWWWWMMMM

R = A or G
Y = C or T
M = A or C
K = G or T
W = A or T
S = C or G
B = C or G or T
D = A or G or T
H = A or C or T
V = A or C or G

Figure 3

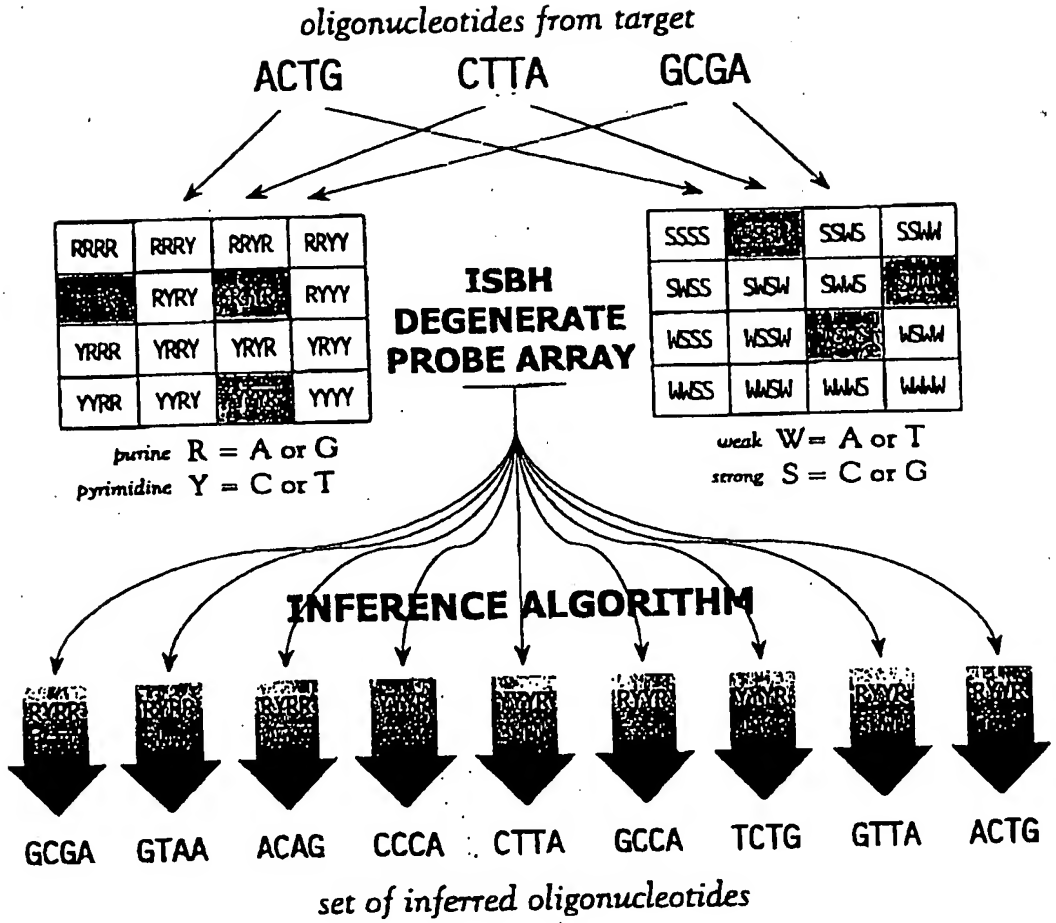

oligonucleotides from target
ACTG
CTTA
GCGA
RRRR
RRRY
RRYR
RRYY
RYRY
RYYY
YRRR
YRRY
YRYR
YRYY
YYRR
YYRY
YYYY
purine R = A or G
pyrimidine Y = C or T
ISBH
DEGENERATE
PROBE ARRAY
SSSS
SSSW
SSWS
SSWW
SWSS
SWSW
SWWS
WSSS
WSSW
WSWW
WWSS
WWSW
WWWS
WWWW
weak W = A or T
strong S = C or G
INFERENCE ALGORITHM
GCGA
GTAA
ACAG
CCCA
CTTA
GCCA
TCTG
GTTA
ACTG
set of inferred oligonucleotides

Figure 4

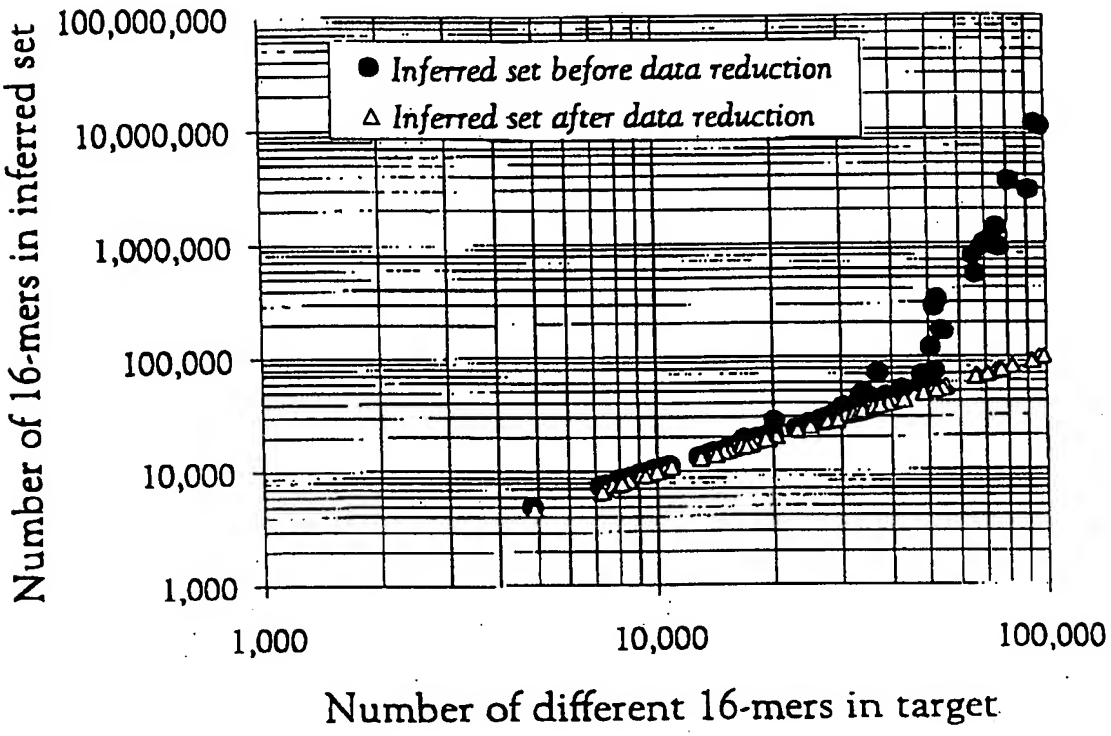

100,000,000
10,000,000
1,000,000
100,000
10,000
1,000
Number of 16-mers in inferred set
Inferred set before data reduction
Inferred set after data reduction
1,000
10,000
100,000
Number of different 16-mers in target

Figure 5

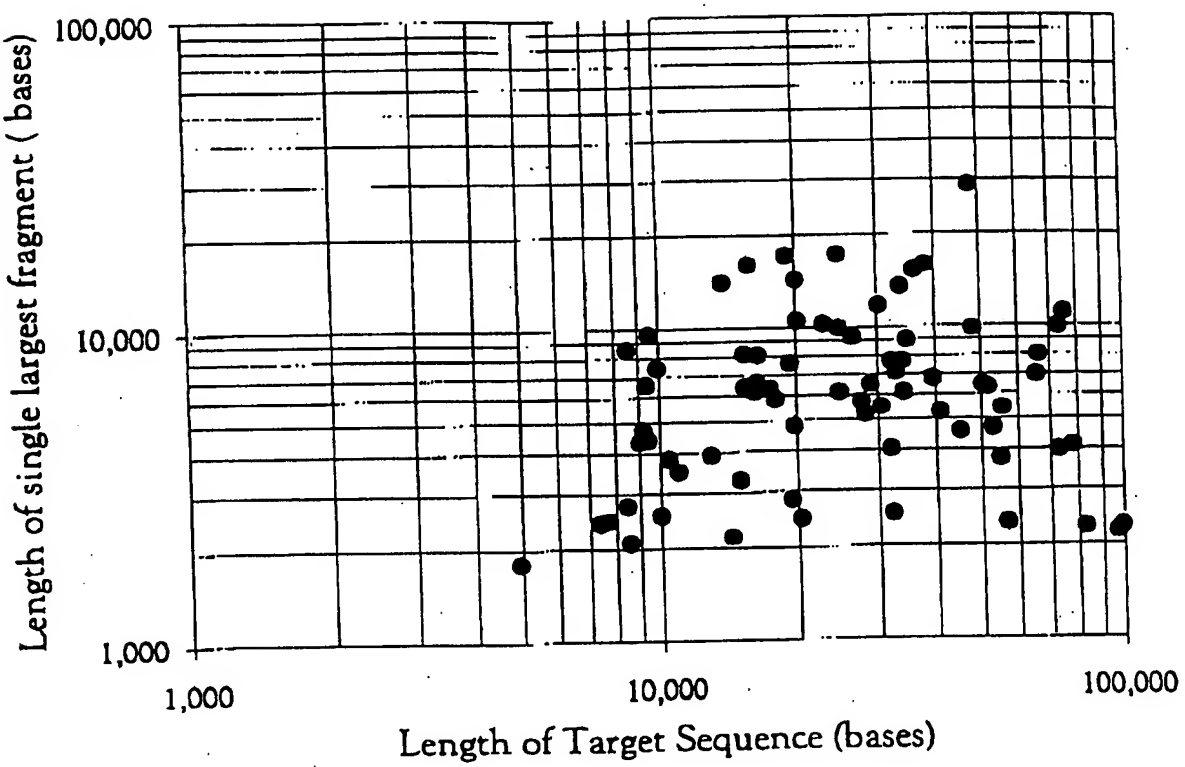

100,000
10,000
1,000
Length of single largest fragment (bases)
1,000
10,000
100,000
Length of Target Sequence (bases)

Figure 6

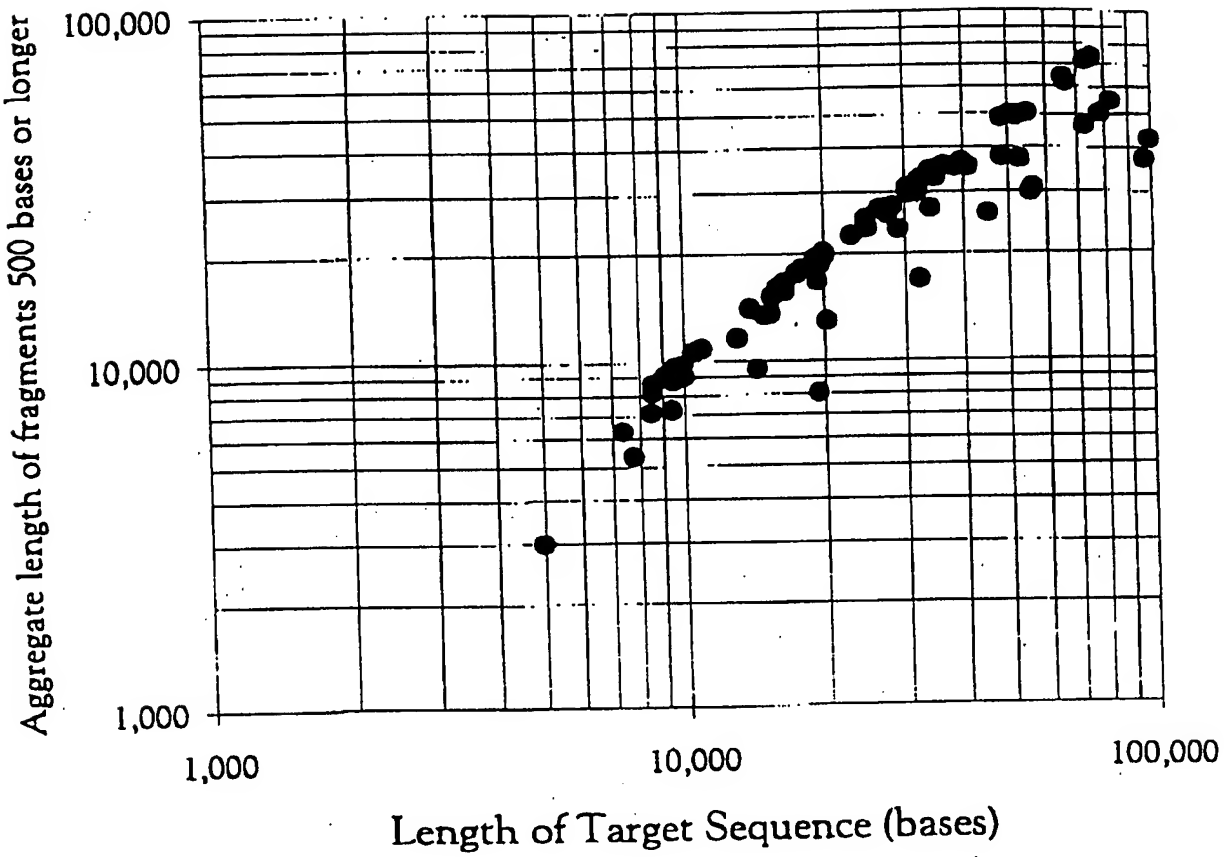
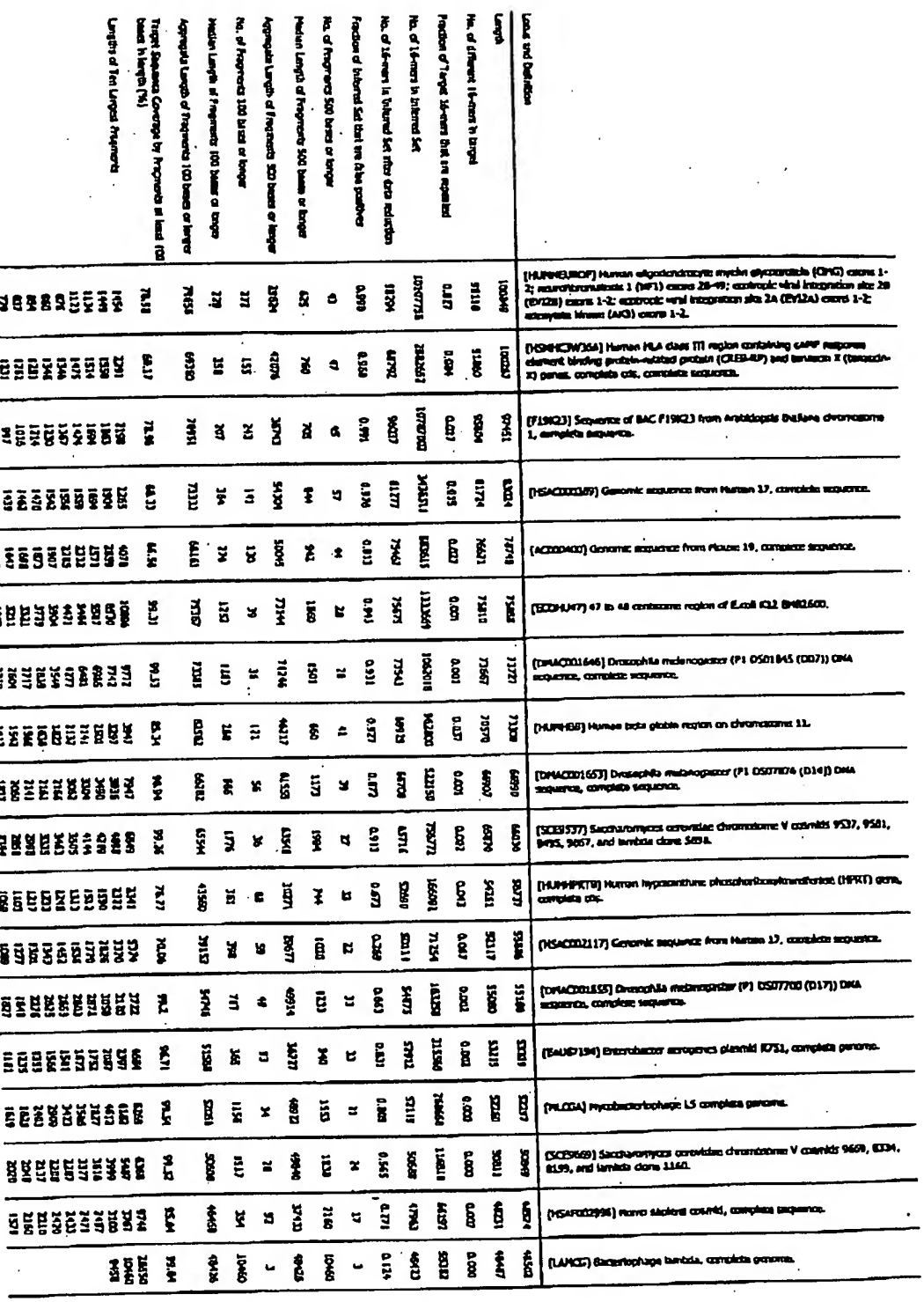
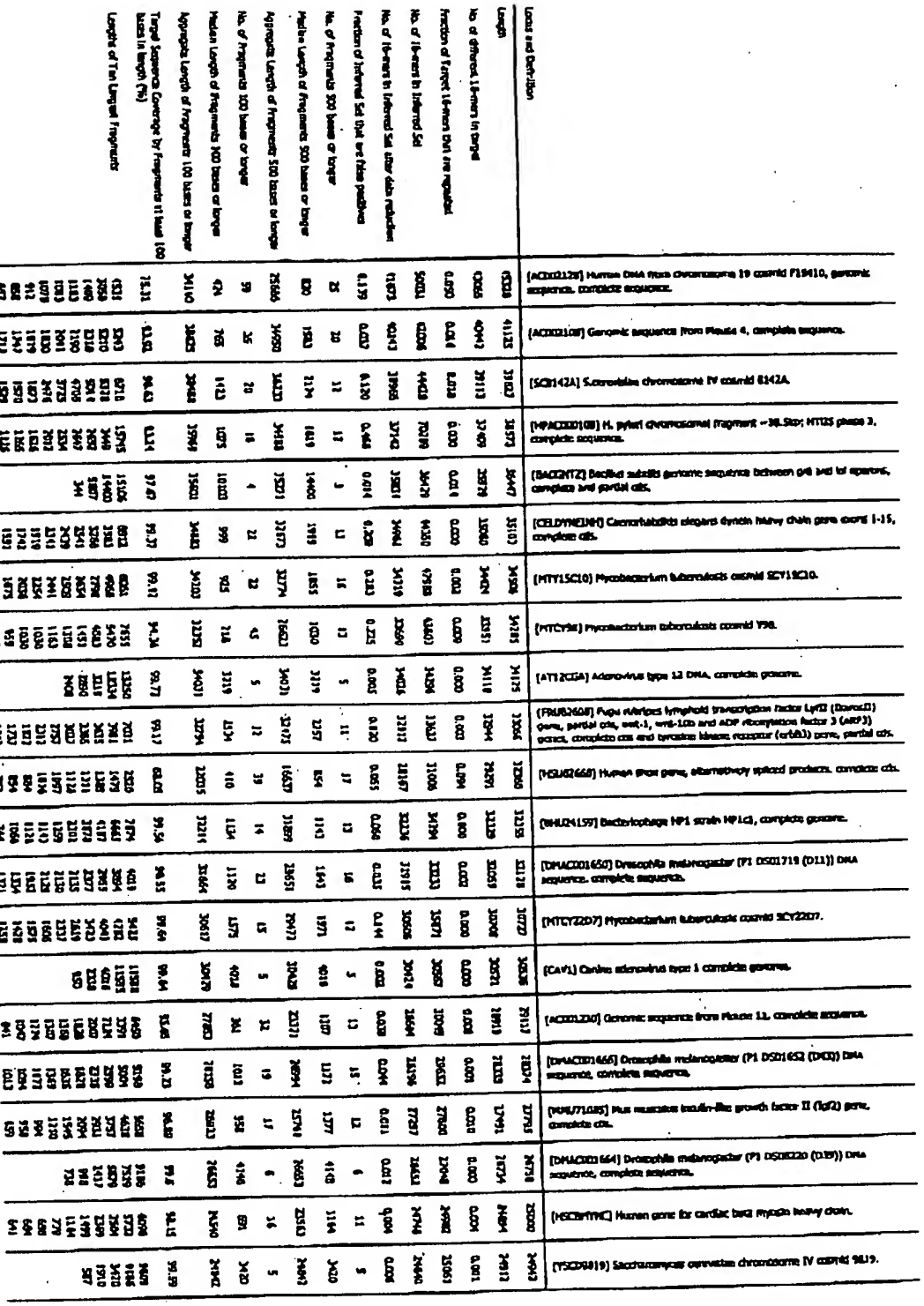

Aggregate length of fragments 500 bases or longer
100,000
10,000
1,000
1,000
10,000
100,000
Length of Target Sequence (bases)

Figure 7



Locus and Definition	Length	No. of Different 16-mers in Target	Fraction of Target 16-mers that are Repeated	No. of 16-mers in Inferred Set	No. of 16-mers in Inferred Set after Data Reduction	Fraction of Inferred Set that are False positives	No. of Fragments 500 bases or longer	Median Length of Fragments 500 bases or longer	Aggregate Length of Fragments 500 bases or longer	No. of Fragments 100 bases or longer	Median Length of Fragments 100 bases or longer	Aggregate Length of Fragments 100 bases or longer	Target Sequence Coverage by Fragments at least 100 bases in length (%)	Lengths of Ten Largest Fragments
[HUMMGUROP] Human oligodendrocyte myelin glycoprotein (OMG) exons 1-2; neurofibromatosis 1 (NF1) exons 28-49; ecotropic viral integration site 2B (EVI2B) exons 1-2; ecotropic viral integration site 2A (EVI2A) exons 1-2; adenylate kinase (AK3) exons 1-2.	100046	59110	0.817	10307718	18294	0.959	625	1934	78818	278	2177	78455	78.18	6454 6446 6446 6446 6446 6446 6446 6446 6446 6446 6446
[HSHHCJW36A] Human HLA class III region containing cAMP response element binding protein-related protein (CREB-RP) and tenascin X (tenascin-X) genes, complete cds, complete sequence.	102080	51860	0.594	2838562	64792	0.958	760	4	42074	158	155	69350	68.17	2261 2261 2261 2261 2261 2261 2261 2261 2261 2261
[F19H23] Sequence of BAC F19H23 from Arabidopsis thaliana chromosome 1, complete sequence.	97451	55804	0.027	10770700	96037	0.991	782	45	36743	207	243	74951	73.96	3158 3158 3158 3158 3158 3158 3158 3158 3158 3158
[HSAC000389] Genomic sequence from Human 17, complete sequence.	83034	81724	0.035	3458511	81277	0.836	57	944	54304	191	394	73111	68.33	3255 3255 3255 3255 3255 3255 3255 3255 3255 3255
[AC000400] Genomic sequence from Mouse 19, complete sequence.	74748	70471	0.027	3058615	75449	0.813	94	943	55005	174	130	69110	64.94	4078 4078 4078 4078 4078 4078 4078 4078 4078 4078
[ECOHUM7] 47 to 48 centisome region of E.coli K12 EMBL600.	71568	71511	0.001	1133569	75473	0.941	28	1869	72344	36	1253	72367	96.31	10886 10886 10886 10886 10886 10886 10886 10886 10886 10886
[DMAC001646] Drosophila melanogaster (P1 DS01845 (D07)) DNA sequence, complete sequence.	71727	71567	0.001	1063018	73543	0.911	28	1501	71046	38	1183	71385	99.53	9713 9713 9713 9713 9713 9713 9713 9713 9713 9713
[HUPHBG] Human beta globin region on chromosome 11.	71308	71578	0.027	942800	69613	0.572	41	650	44317	121	158	65783	65.34	3964 3964 3964 3964 3964 3964 3964 3964 3964 3964
[DMAC001653] Drosophila melanogaster (P1 DS07876 (D14)) DNA sequence, complete sequence.	69969	69902	0.001	922150	65708	0.873	36	1173	61453	56	196	66282	94.94	7047 7047 7047 7047 7047 7047 7047 7047 7047 7047
[SCE35737] Saccharomyces cerevisiae chromosome V cosmids 9537, 9581, 8495, 9057, and lambda clone 5898.	66196	65804	0.021	750772	64714	0.913	27	1984	63548	36	1776	65594	95.36	4288 4288 4288 4288 4288 4288 4288 4288 4288 4288
[HUMHPRTB] Human hypoxanthine phosphoribosyltransferase (HPRT) gene, complete cds.	50027	54251	0.043	1669091	52560	0.673	33	744	35271	83	1321	43950	74.77	1968 1968 1968 1968 1968 1968 1968 1968 1968 1968
[HSAC002117] Genomic sequence from Human 17, complete sequence.	53896	53217	0.067	715754	52311	0.269	22	1020	35677	58	758	36153	70.04	6888 6888 6888 6888 6888 6888 6888 6888 6888 6888
[DMAC001655] Drosophila melanogaster (P1 DS07700 (D17)) DNA sequence, complete sequence.	51148	51080	0.003	1613258	54472	0.963	11	1133	46934	49	717	54746	98.2	1827 1827 1827 1827 1827 1827 1827 1827 1827 1827
[EAU07194] Enterobacter aerogenes plasmid IO751, complete genome.	53389	53115	0.003	715566	57912	0.831	13	940	34027	56	1367	53858	96.71	1818 1818 1818 1818 1818 1818 1818 1818 1818 1818
[MLCGA] Mycobacteriophage L5 complete genome.	52237	52220	0.000	748464	53111	0.808	11	1153	46812	54	1158	52018	98.54	6368 6368 6368 6368 6368 6368 6368 6368 6368 6368
[SCE39669] Saccharomyces cerevisiae chromosome V cosmids 9669, 8334, 8199, and lambda clone 1160.	50069	50081	0.000	1186118	56458	0.565	24	1133	49648	28	1517	50308	94.12	5202 5202 5202 5202 5202 5202 5202 5202 5202 5202
[HSAF002996] Homo sapiens cosmid, complete sequence.	48824	48521	0.000	845857	47843	0.721	17	1160	37513	52	1254	44668	95.64	1578 1578 1578 1578 1578 1578 1578 1578 1578 1578
[LAMCG] Bacteriophage lambda, complete genome.	48502	48477	0.000	838338	46423	0.124	J	10460	48436	J	10460	48436	99.84	48502 48502 48502 48502 48502 48502 48502 48502 48502 48502

Figure 8 (1 of 4)



Locus and Definition	Length	No. of different Libraries in Sample	Fraction of Target Libraries that are represented	No. of Libraries in Inferred Set	No. of Libraries in Inferred Set after data reduction	Fraction of Inferred Set that are False positives	No. of Fragments 500 bases or longer	Median Length of Fragments 500 bases or longer	Aggregate Length of Fragments 500 bases or longer	No. of Fragments 100 bases or longer	Median Length of Fragments 100 bases or longer	Aggregate Length of Fragments 100 bases or longer	Target Sequence Coverage by Fragments at least 100 bases in length (%)	Length of Tiled Longest Fragments
[AC003128] Human DNA from chromosome 19 cosmid F19H10, genomic sequence, complete sequence.	41538	13866	0.000	5	0.000	0.000	800	1000	800000	1000	1000	1000000	78.31	341140
[AC003108] Genomic sequence from Phage 4, complete sequence.	41132	40443	0.014	0.000	40443	0.000	1583	1000	1583000	1665	1000	1665000	83.01	338525
[SC8142A] S.cerevisiae chromosome IV cosmid 8142A.	39167	28113	0.078	0.000	44403	0.000	2114	1000	2114000	2113	1000	2113000	96.63	339488
[MPAC000108] H. pylori chromosomal fragment ~38.5kb; HTGS phase 3, complete sequence.	35573	33403	0.000	0.000	78198	0.000	1819	1000	1819000	1075	1000	1075000	83.1	339649
[BACGNTZ] Bacillus subtilis genomic sequence between gsk and tal spoVC, complete and partial cds.	35447	32878	0.018	0.000	34530	0.000	1400	1000	1400000	1033	1000	1033000	97.67	339447
[CELDYNEUH] Caenorhabditis elegans dynein heavy chain gene exons 1-15, complete cds.	35193	33560	0.000	0.000	34564	0.000	1319	1000	1319000	999	1000	1000000	79.37	339400
[MTY15C10] Mycobacterium tuberculosis cosmid SCY15C10.	34596	34434	0.000	0.000	69588	0.133	1855	1000	1855000	1004	1000	1004000	99.11	340100
[MTCY98] Mycobacterium tuberculosis cosmid Y98.	34185	33351	0.009	0.000	63463	0.222	1830	1000	1830000	1003	1000	1003000	94.34	339253
[AT12CGA] Adeno-Virus type 12 DNA, complete genome.	34115	34118	0.000	0.000	34284	0.000	1119	1000	1119000	1119	1000	1119000	99.72	340401
[FRUR2608] Fugu rubripes lymphoid transcription factor Lyf2 (Dorsal) gene, partial cds; ser1-1, ser1-1Gb and ADP ribosylation factor 3 (ARF3) genes, complete cds and tyrosine kinase receptor (erbB3) gene, partial cds.	33904	33903	0.000	0.000	34284	0.000	1257	1000	1257000	1274	1000	1274000	99.17	337472
[HSU82668] Human ikaros gene, alternatively spliced products, complete cds.	33868	31008	0.094	0.000	74291	0.095	854	1000	854000	1005	1000	1005000	68.05	220355
[BHU24159] Bacteriophage HP1 strain HP1c1, complete genome.	33358	33136	0.000	0.000	34594	0.056	1143	1000	1143000	1134	1000	1134000	99.56	338214
[DMAC001650] Drosophila melanogaster (P1 DS01719 (D11)) DNA sequence, complete sequence.	33358	33333	0.000	0.000	71915	0.125	1143	1000	1143000	1130	1000	1130000	98.55	338464
[MTCY22D7] Mycobacterium tuberculosis cosmid SCY22D7.	33722	33698	0.000	0.000	33671	0.094	1171	1000	1171000	1172	1000	1172000	97.44	339617
[CAV1] Canine adenovirus type 1 complete genome.	33636	33621	0.000	0.000	33682	0.000	4018	1000	4018000	4024	1000	4024000	98.84	339439
[AC003106] Genomic sequence from Phage 11, complete sequence.	33611	33618	0.000	0.000	34694	0.000	1107	1000	1107000	1106	1000	1106000	83.46	272823
[DMAC001466] Drosophila melanogaster (P1 DS01652 (D123)) DNA sequence, complete sequence.	33474	31433	0.000	0.000	34594	0.094	1127	1000	1127000	1094	1000	1094000	94.33	271338
[MMU71085] Mus musculus insulin-like growth factor II (Igf2) gene, complete cds.	33715	32920	0.000	0.010	27257	0.011	1277	1000	1277000	1558	1000	1558000	96.89	338521
[DMAC001664] Drosophila melanogaster (P1 DS00220 (D39)) DNA sequence, complete sequence.	33714	32948	0.000	0.000	34353	0.011	1418	1000	1418000	1436	1000	1436000	79.8	269653
[HSCBHTHC] Human gene for cardiac beta myosin heavy chain.	33500	34984	0.004	0.004	34982	0.004	1194	1000	1194000	1194	1000	1194000	98.15	340596
[YSCD9819] Saccharomyces cerevisiae chromosome IV cosmid 9819.	34432	33961	0.001	0.005	34448	0.005	1420	1000	1420000	1420	1000	1420000	98.58	340482

Figure 8 (2 of 4)

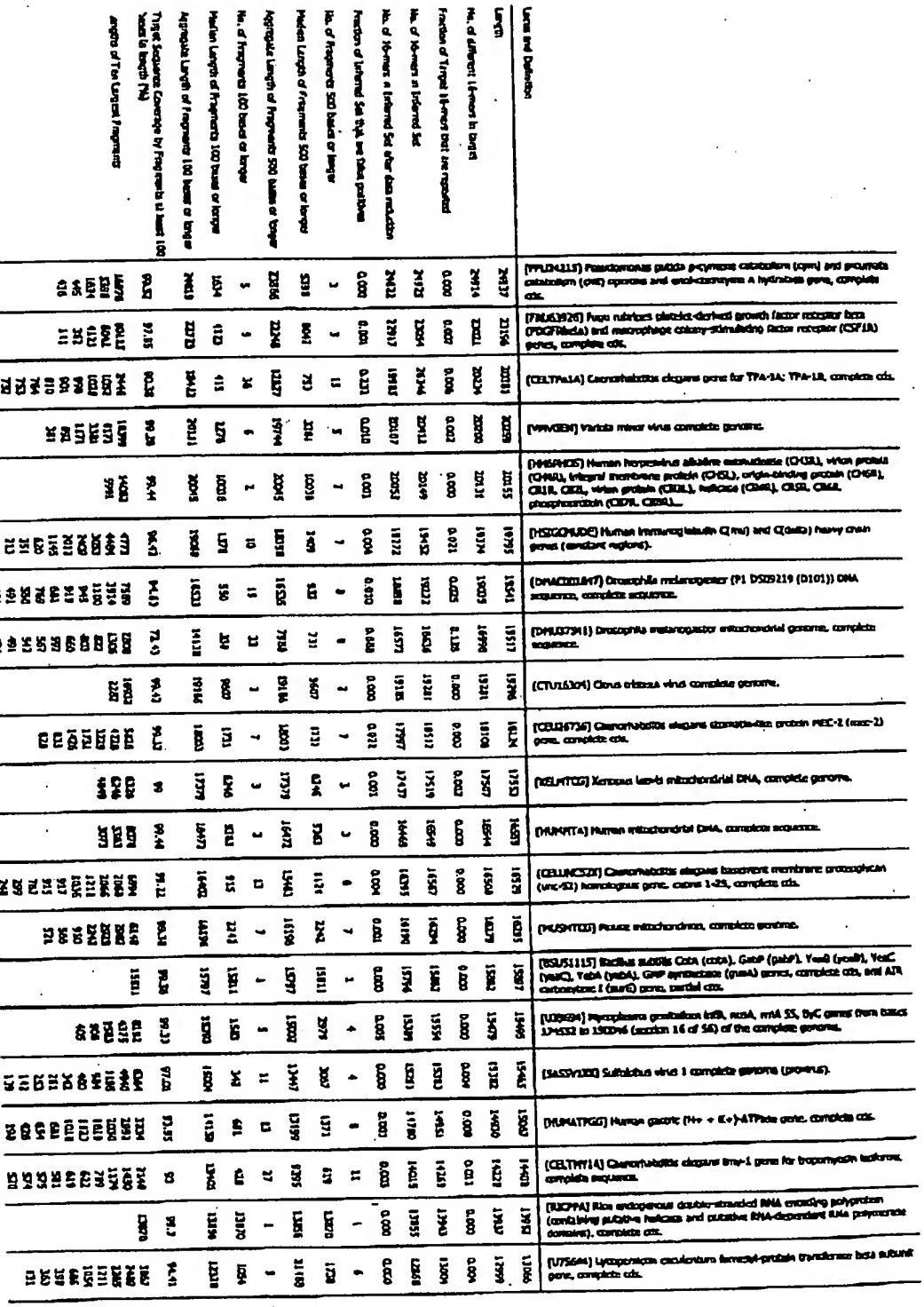
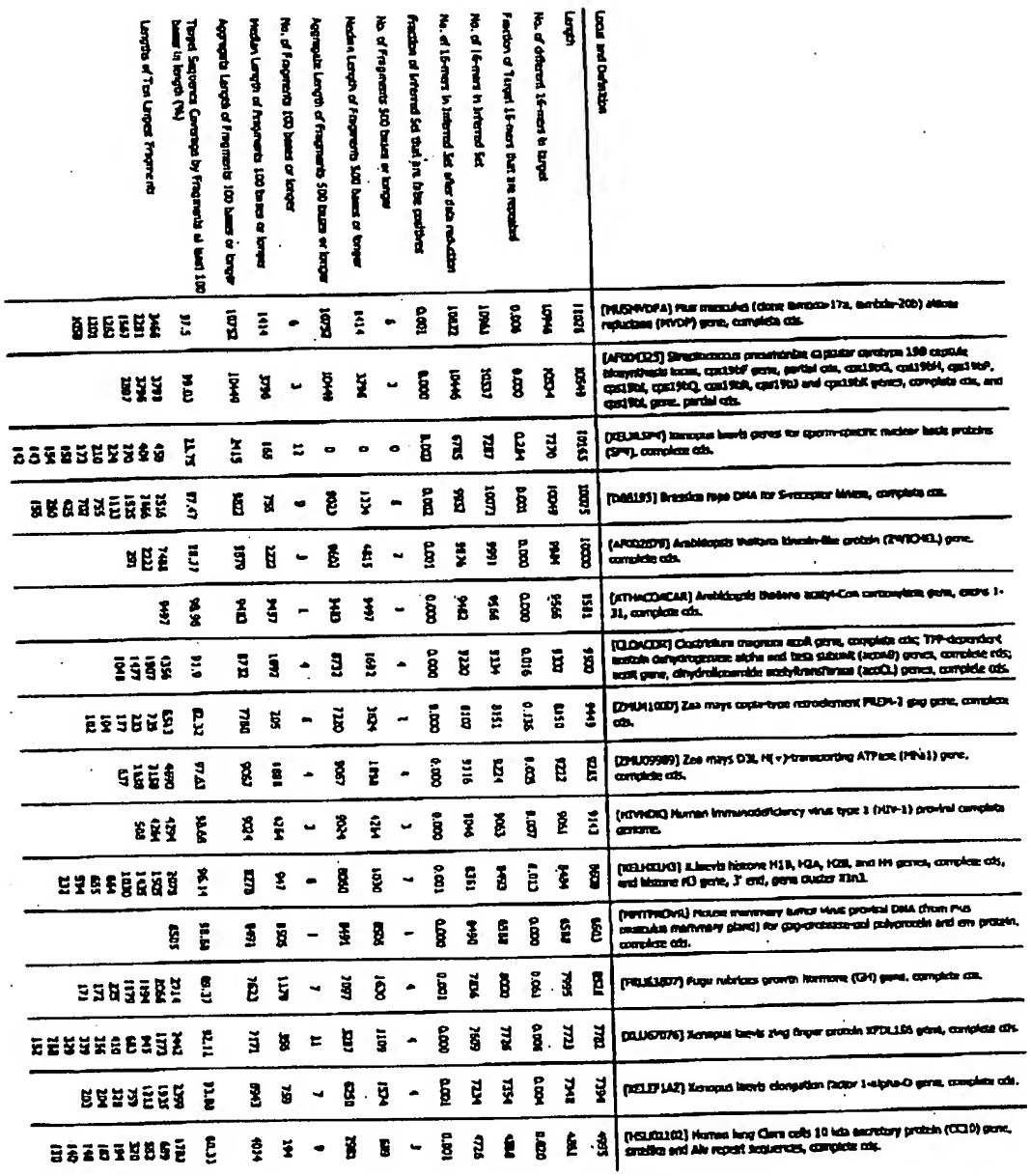


Figure 8 (3 of 4)



Locus and Definition	Length	No. of different 16-mers in Target	Fraction of Target 16-mers that are repeated	No. of 16-mers in Interval Set	No. of 16-mers in Interval Set after Stale reduction	Fraction of Interval Set that is in bite positions	No. of Fragments 500 bases or longer	Median Length of Fragments 500 bases or longer	Aggregate Length of Fragments 500 bases or longer	No. of Fragments 100 bases or longer	Median Length of Fragments 100 bases or longer	Aggregate Length of Fragments 100 bases or longer	Target Sequence Coverage by Fragments at least 100 bases in length (%)	Lengths of Ten Largest Fragments
[MUSHMDFA] Mus musculus (clone Bamboo-17a, Bamboo-20b) dihydrofolate reductase (MMDHF) gene, complete cds.	11826	10946	0.000	10784	10682	0.001	6	1414	10703	9	1414	10733	87.5	3466 3231 3163 3150 3028
[AF000325] Streptococcus pneumoniae capsular serotype 19B capsule biosynthesis locus, cps19BF gene, partial cds; cps19BG, cps19BH, cps19BP, cps19BI, cps19BQ, cps19BR, cps19BJ and cps19BK genes, complete cds; and cps19BL gene, partial cds.	10549	9804	0.000	10446	10337	0.000	3	3294	10048	3	3294	10449	94.03	3788 3294 3287
[XELJRLSP4] Xenopus laevis genes for sperm-specific nuclear basic proteins (SP4), complete cds.	10165	7270	0.284	9735	7287	0.000	0	0	0	11	169	1415	13.76	69 68 66 64 62 61 60 59 58 57 56 55 54 53 52 51 50 49 48 47 46 45 44 43 42 41 40 39 38 37 36 35 34 33 32 31 30 29 28 27 26 25 24 23 22 21 20 19 18 17 16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1
[D88193] Brassica rapa DNA for S-receptor kinase, complete cds.	10005	10049	0.001	9583	10077	0.001	8	1134	8003	9	755	8823	87.47	3516 3466 3333 3331 3111 3111 2755 2732 2683 2683 2683